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Protein-associated pigments that accumulate in the brunescent eye lens

Identification of a quinoline derivative

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Abstract

Brunescent (dark brown) cataract is particularly prevalent in the tropics. Enzymatic digestion of the insoluble protein fraction of brunescent cataractous eye lenses from India, followed by high performance liquid chromatographic separation of the pigments and spectroscopic investigations, have led to the identification of one of the pigments as 4-hydroxyquinoline-3-[α-aminoacetic acid] (compound A). The 4-hydroxyquinoline moiety is shown to be a photodynamic agent that generates $O_2^{\cdot-}$ and leads to protein crosslinking. This suggests that the compound A may play a long-term deleterious role in situ in the lens.

Key words: Brown cataract lens; Tryptophan oxidative modification; 4-Hydroxyquinoline-3-[α-aminoacetic acid]; Endogenous photodynamic ability; Protein covalent crosslinking

1. Introduction

The normal mammalian eye lens is a transparent body made up of long enucleated fiber-shaped cells of epithelial origin which, during differentiation, rid themselves of large organelles that are likely to scatter light, and over-express structural proteins, called crystallins, to very high intracellular concentrations (200–500 mg/ml). These highly concentrated crystallin solutions minimize light scattering through short-range ordering of molecules into a glass-like state. A reduction in light-scattering is also achieved through regular intercellular stacking of the hexagonally cross-sectioned fiber cells. Thus, the lens owes its transparency, as well as its other optical properties (such as the existence of a smooth radial refractive gradient), to the ordered organization of its constituents [1].

Changes in such molecular ordering, arising from protein conformational changes, aggregation and precipitation, bring about an increase in light scattering through particulate protein matter, and through discontinuities generated in the radial refractive gradient. Such changes tend to be permanent, since there is no turnover of molecules in enucleated cells [2]. With aging and chronic oxidative stress, crystallins undergo covalent chemical modifications leading to the formation of high molecular weight aggregates, insoluble protein particles and increased pigmentation [3–7]. Oxidation is believed to result largely from photochemical processes that convert aromatic amino acids to potent photosensitizers or simple chromophores which absorb near-UV and low wavelength visible light. The generation of reactive oxygen species by some of these chromophores is thought to lead to widespread protein oxidation. Little is known, however, about either the covalent nature or age-dependent accumulation of the pigments that are generated, or about the physiological roles that some of these might have in the lens.

One form of cataract that is particularly prevalent in the tropical regions of the world is brunescent or dark brown cataract. Epidemiological studies have implicated this higher incidence of brunescent cataract to the increased levels of insolation in the tropics [8–11]. Brunescent cataract is characterized by high amounts (as much as 70% of the total protein) of insoluble proteins in the lens, and pronounced absorption and emission in the near-UV and visible regions of the spectrum [12–14].

Evidence is increasingly gathering to support the suggestion that tryptophan (Trp) metabolites and photoproducts constitute at least a fraction of the pigments generated in the lens [12,15–20]. Some of these that have been identified are kynurenine (Ky), *N*-formylkynurenine (NFK), 3-hydroxykynurenine (3HK) and its glucoside (3HKG), β-carbolines, and anthranilic acid derivatives [15,18–20,22,23].

In this paper, we report the isolation and structural characterization of a novel and hitherto unreported pigment that accumulates in brunescent cataract lenses excised from human patients, namely 4-hydroxyquinoline-3-[α-aminoacetic acid] or compound A (see Fig. 3). We

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further show that the 4-hydroxyquinoline moiety of this compound is photodynamic, and generates reactive oxygen species upon irradiation with UVA light, leading to protein crosslinking. This suggests that compound A may play a long-term deleterious role in situ in the lens.

2. Materials and methods

2.1. Isolation of the pigments

Brunescent (very dark brown) human lenses excised through cataract surgery of elderly (50–55 year old) patients, obtained from the L.V Prasad Eye Institute, Hyderabad, India, were homogenized in water and centrifuged at 10,000 × *g* for 15 min at 4°C. The resulting dark brown pellet was solubilized by incubation at 37°C of the pellet with an aqueous solution of pronase (Boehringer; 1 mg pronase for every 100 mg pellet) at neutral pH, so as not to decompose the pigments. After 24 h, a further 0.5 mg pronase per 100 mg pellet weight was added. After 48 h, the enzyme leucine aminopeptidase (Sigma; 0.1 mg/100 mg weight of pellet) was added and the digestion ended after 60 h by heating for 2 min in a boiling water bath. The solution was chromatographed on PD-10 desalting columns of Sephadex G-25M to remove the enzymes; the mixture of hydrolyzed amino acids and small peptides, generated through protease action, was eluted with water and then re-chromatographed on a Waters C_{18} reverse-phase column (30 cm × 7.8 mm i.d), using a Pharmacia HPLC system. The mobile phase consisted of 5% methanol, run isocratically at 1.5 ml/min with absorbance monitored at 400 nm. The purity of the eluted fractions was checked on precoated Kieselgel TLC plates (silica gel 60 F_{254}; supplied by Merck). The solvent system used was *n*-butanol:acetic acid:water (8:1:2) and the spots were visualised by iodine staining or with ninhydrin.

2.2. Spectral measurements

Absorption spectra were recorded on a Hitachi model 330 spectrophotometer and fluorescence spectra on a Hitachi model F-4000 spectrofluorimeter. One-dimensional NMR spectra were recorded on a AM-300 MHz Bruker machine. Fast atom bombardment (FAB) mass spectra were recorded on a JEOL SX 102/DA-6000 mass spectrometer/data system using xenon (6 kV, 10 mA) as the FAB gas, an accelerating voltage of 10 kV and *m*-nitrobenzyl alcohol (NBA) as the matrix.

2.3. Studies on lens proteins

Bovine lenses (8–10) were decapsulated and homogenized in 0.1 M Tris buffer, pH 7.4, containing 0.5 M NaCl, 1 mM EDTA and 0.1% NaN_3. The insoluble protein fraction was removed by centrifugation (30,000 × *g*, 30 min) and the supernatant was chromatographed on a Sephacryl S-200 gel filtration column (0.04 × 1.8 m) in order to separate the individual α-, β_H-, β_L- and γ-crystallins. Each of the crystallins was dialysed repeatedly against water and stored in the lyophilized form. γ-Crystallin was used in the photosensitization studies. Solutions were taken in a fluorescence cuvette (1 × 1 × 4 cm) and placed in the sample compartment of a Hitachi spectrofluorimeter (model F-4000) under conditions of constant stirring, and irradiated with light corresponding to the absorption band of the photosensitizers included in the protein solution, using a monochromator set at a bandwidth of 20 nm to restrict light originating from a broad-emitting 150 W Xe arc lamp source. The light intensity falling on the sample was estimated to be around 2.0–2.4 $mW \cdot cm^{-2}$ (approximately 10^{14} photons $\cdot s^{-1}$) in the 295–335 nm region, and 3.0 $mW \cdot cm^{-2}$ in the 360–365 nm range. Electrophoresis was done on 12.5% polyacrylamide gels, using the Pharmacia Phastsystem assembly. Protein samples were boiled with sodium dodecyl sulfate (SDS) solution containing β-mercaptoethanol, so as to disaggregate the protein and to detect non-disulfide crosslinks.

2.4. Reactive oxygen species

The superoxide anion ($O^{\bullet}_2^-$) was detected using the cytochrome *c* reduction method [24]. Solutions of photosensitizers were illuminated in the presence of ferricytochrome *c* in 50 mM phosphate buffer, pH 7.4, and the reduction monitored spectrophotometrically at 550 nm, using ε_{500} = 20,000 $M^{-1} \cdot cm^{-1}$ for the reduced-oxidized cytochrome *c* [25]. A concentration of 20 μM was used throughout. The generation of hydroxyl radicals (•OH) was assayed by the method of Halliwell et al. [26], which involves incubation of the reaction mixture with 2–3 mM deoxyribose at 37°C. Color development is normally achieved by the addition of a 1% solution of thiobarbituric acid (TBA) in 50 mM NaOH, followed by heating of the tubes at 100°C for 10–20 min and cooling. The resulting TBA adduct was measured by its absorbance at 532 nm.

3. Results and discussion

3.1. Isolation and purification of fluorophores

The enzyme-digested lenticular material was first chromatographed on a Sephadex G-25 column to remove all the enzyme, and the small molecular eluate fractions were separated and purified using reverse-phase HPLC. Four eluates absorbing at 400 nm were collected from the column at retention times of 6.8, 9.9, 13.5 and 21.3 min. Three of these (9.9, 13.5 and 21.3 min fractions) were re-chromatographed on the same system and purified to homogeneity. Attempts are being made to separate the 6.8 min peak into its individual components. Analysis of the compound eluting at 9.9 min indicates it to be racemic tyrosine. (NMR and mass spectra of the purified fraction that eluted at 9.9 min revealed it to be DL-tyrosine. The extent of racemization was estimated, by measuring its optical rotatory dispersion, to

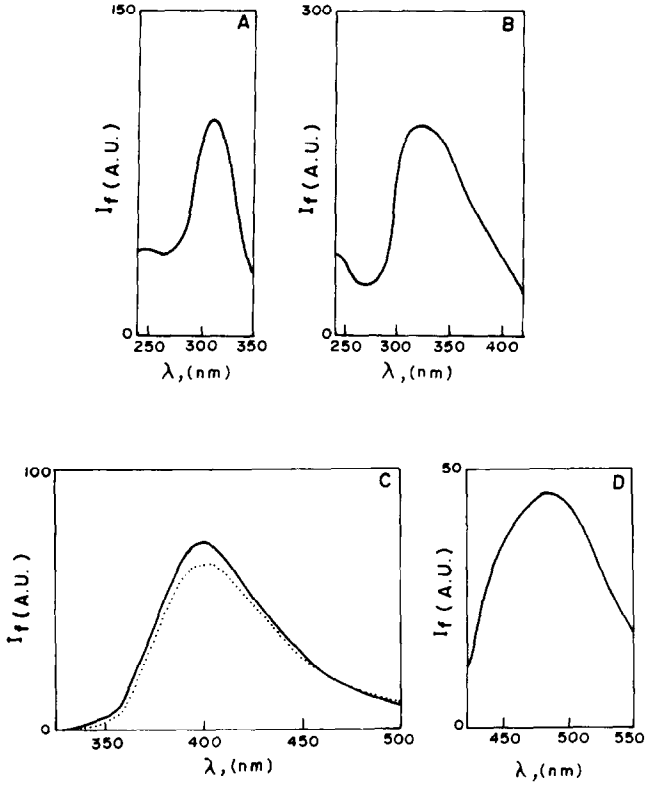

Fig. 1. Fluorescence analysis of the 21.3 min fraction: (A), excitation spectra with the emission monochromator set at 400 nm; (B) excitation spectra with the emission monochromator set at 480 nm; (C) emission profile upon excitation at 313 nm (—) and 323 nm (·····); (D) emission profile upon excitation at 400 nm. A.U refers to arbitrary units.

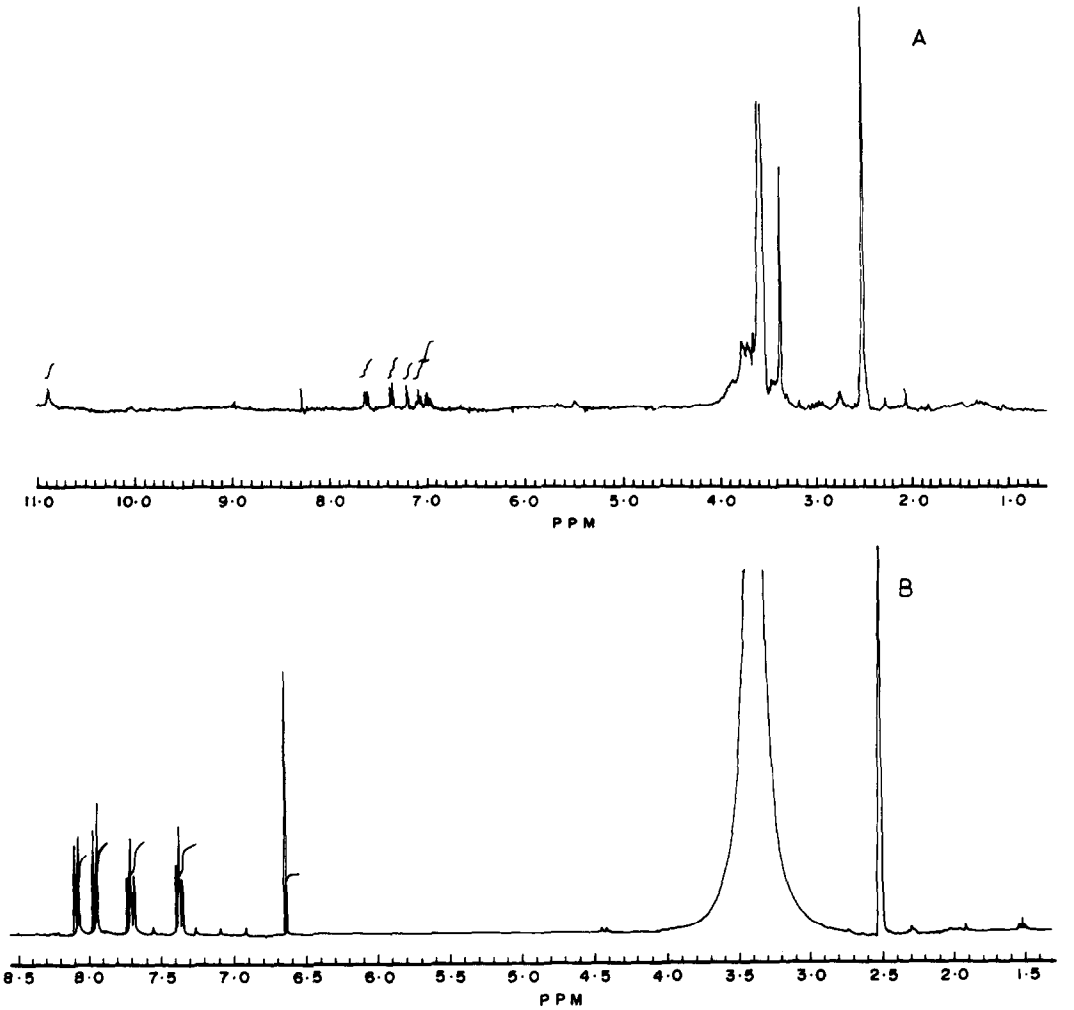

Fig. 2. 300 MHz ^{1}H NMR spectrum of (A) the 21.3 min fraction in DMSO and (B) authentic kynurenic acid in DMSO.

be around 10%. The absorption displayed around 400 nm is because of its occurrence as a 1:1 dimeric complex in the quinhydrone form [27]. Age-dependent racemization in long-lived protein molecules is known, and has been reported in cataract lenses with aspartic acid and other amino acid residues [28–30]. The structure elucidation of the 13.6 min fraction is currently in progress.) We report here the analysis of the fraction corresponding to the peak with a retention time of 21.3 min.

3.2. Structure of the 21.3 min fraction

Excitation spectral analysis of the 21.3 min fraction, with the emission monochromator set at 400 nm, shows a maxima at 313 nm (Fig. 1A) while that with the monochromator at 480 nm shows a broad peak at 323 nm (Fig. 1B). The emission profiles obtained from excitations at 313 and 323 nm are shown in Fig. 1C, while that arising from excitation at 400 nm is shown in Fig. 1D.

The NMR spectrum of a DMSO-d_6 solution of this fraction is presented in Fig. 2A. The spectrum may be grouped into five groups of signals: two sets of doublets on the low field, two sets of triplets (or rather multiplets) around 7 ppm, and a singlet at 7.2 ppm. Such a spectrum is characteristic of heterocyclic species, the proton spectra of which are seriously complicated by the overlapping of spectral multiplets. Through a process of both looking into the chemistry of modification of Trp, as well as comparison of the spectrum with a series of heterocyclic species, it was possible to assign the spectrum to a quinoline derivative. The presence of a singlet in the spectrum implies that the heterocyclic ring of quinoline is substituted at two positions. This suggests two possibilities, as shown in Fig. 3. One of these molecules is kynurenic acid (KUA), which has been implicated in the oxidative pathway of Trp. However, the possibility of this fraction being KUA was ruled out by the following. Comparison with the NMR spectrum of an authentic KUA sample (Fig. 2B) shows that while the same sets of signals are present, their chemical shifts are quite different. Specifically, the position of the singlet can be used to resolve the issue, since it originates from the heterocyclic ring of quinoline and its chemical shift would be determined by

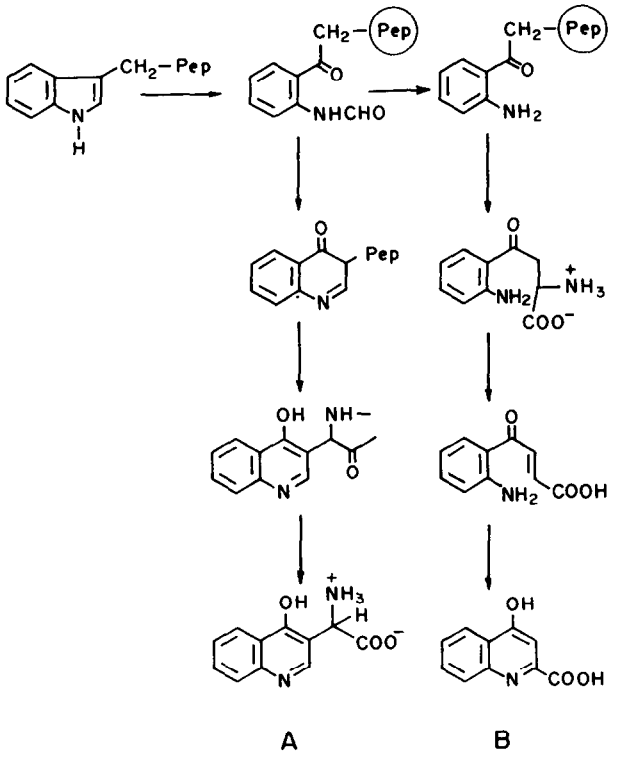

Fig. 3. Proposed mechanism for the formation of (A) 4-hydroxyquinoline-3-[α-aminoacetic acid] and (B) kynurenic acid (KUA). Pep refers to the peptide (backbone) chain.

the presence of its neighbours. The singlet in the case of KUA is at 6.5 ppm while the one in the fraction being analysed appears at 7.2 ppm. The CH proton (singlet) from compound A would be expected to occur at a higher ppm value, being at the α-position of the ring nitrogen. (Normally such a proton would appear further downfield but the value of the chemical shift is lower in this case, which could be attributed to the possibility of tautomeric forms of compound A.) Thus the fraction under consideration seems to be (A) 4-hydroxyquinoline-3-[α-aminoacetic acid] (or 3-[α-glycyl]-4-hydroxyquinoline), and not (B) kynurenic acid (or 4-hydroxyquinoline-2-carboxylic acid).

Further confirmation of the structure of this molecule comes from the FAB mass spectra shown in Fig. 4. The *m/z* at 218 matches the calculated molecular weight and may be attributed to the species, M^+, which is commonly observed in the positive-ion FAB of organic compounds [31]. Positive-ion FAB can yield cationated species such as Na^+ adducts; the mass at 240 could be one such adduct. Also, 4-hydroxyquinoline is known to show absorption bands at 315 and 328 nm [32], much like the excitation bands obtained in the present case (Fig. 1A and B).

According to the mechanism proposed in Fig. 3, it is evident that this structure should have existed prior to the enzymatic cleavage of the peptide bond and that the ring closure may not have happened as a consequence of such a bond cleavage, as it would be for molecule B (KUA). In this event, the possibility of finding KUA in the protein would appear to be remote, since the formation of KUA requires that the α-carbon atom of the amino acid be available for ring closure after cleavage of the peptide bond. The presence of KUA in any lens is yet to be confirmed, even though it can arise through the oxidative pathway. Thus, if KUA were to be detected in the lens, it would arise through the oxidation of free Trp, and not from Trp residues present in the protein.

Irradiation of Trp and of NFK at pH 7.3, using sunlight or a 'daylight lamp' has been shown to give 4-hydroxyquinoline as one of the products [32]. Thus, we surmise that the compound A seen in the brown insoluble portion of the cataract lens from India is the result of long-term photochemical modification of the Trp residues of the lens proteins, although its accumulation from 'dark' reactions is not to be dismissed. Indeed, the parent compound, i.e. 4-hydroxyquinoline, has been detected as a metabolite of kynurenine in silkworms [33]

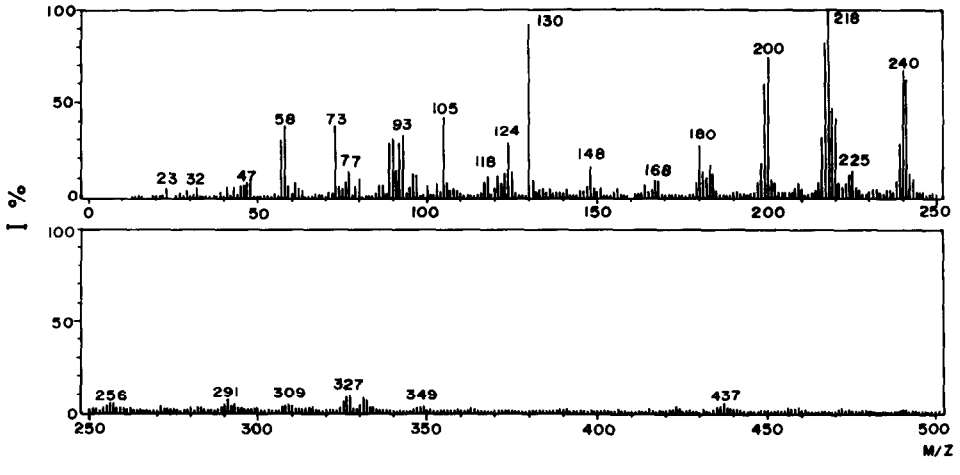

Fig. 4. Fast atom bombardment (FAB) mass spectrum of the 21.3 min fraction with *m/z* at 218. FAB gas was xenon (6 kV, 10 mA), accelerating voltage was 10 kV and the spectrum was recorded at room temperature.

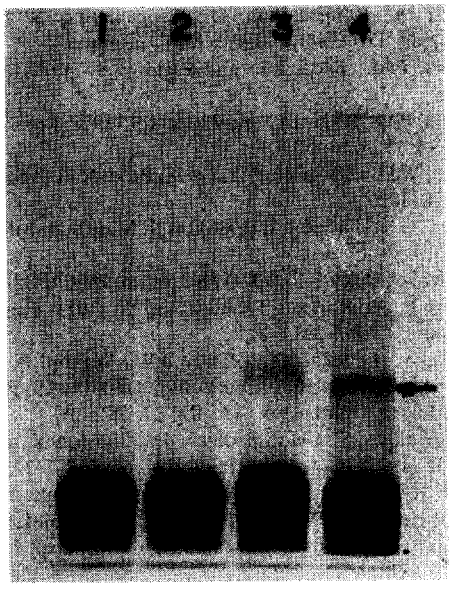

Fig. 5. SDS-PAGE patterns of 1 mg/ml solutions of bovine γ-crystallin, irradiated for a period of 1 h each, using a Hitachi F-4000 spectrofluorimeter light source with monochromator set at the respective wavelengths, slit 20 nm. Lane 1, γ-crystallin irradiated at 325 nm; lane 2, γ-crystallin irradiated at 332 nm; lane 3, γ-crystallin irradiated at 325 nm in the presence of 1 mM 4-hydroxyquinoline; lane 4, γ-crystallin irradiated at 332 nm in the presence of 1 mM kynurenic acid. The arrow points to higher molecular weight products.

and also found in mitochondria of rat liver [34]. To the best of our knowledge [35], ours is the first report of any quinoline derivative being found in the lens.

3.3. *Photodynamic properties of the parent 4-hydroxyquinoline*

Many of the pigments that accumulate in the lens extend the absorption range from the UVB into the UVA and even the visible range. Apart from the color bias that they introduce to the transmission of light, some of them can also act as photodynamic agents which, upon absorption of UVA and visible light, cause sensitized covalent chemical changes in the substrate molecules through the generation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion radical ($O^{\bullet}_2^-$), hydroxyl radical ($^{\bullet}OH$) or peroxide (H_2O_2). We have shown earlier that, among the Trp oxidation products, NFK and KUA are particularly active photodynamic agents, while Ky and 3HK are benign [36]; indeed, 3HK might even be an endogenous antioxidant [37]. In this connection, it is of interest to investigate whether compound A can act as a photodynamic agent and cause further damage to the lens.

Since the amount of compound A purified and studied was in limiting amounts (2 mg isolated from 1650 mg of insoluble wet material from 9 lenses), we could not study its photodynamics directly. As a close approximation, the photosensitization behavior of 4-hydroxyquinoline was studied, since the basic moiety of this parent compound is the same as that in compound A. This was compared with the photosensitization behaviour of KUA. Both these samples were seen not to produce $^{\bullet}OH$ radicals. However, these samples are quite effective in generating $O^{\bullet}_2^-$ radicals upon irradiation with light corresponding to their excitation maxima. No cytochrome *c* reduction was noted in the reaction mixture kept in the dark.

Fig. 5 shows assays of the photodynamic ability of 4-hydroxyquinoline and KUA to produce non-disulfide covalent crosslinks in γ-crystallin molecules, which constitute the major fraction of the core region of mammalian eye lenses. Higher molecular weight bands are seen in lanes 3 and 4, indicating the presence of covalent non-disulfide crosslinking of γ-crystallin. This result suggests that the 4-hydroxyquinoline moiety of compound A can act as a sensitizer (just as KUA does), active in UVA light, and promote covalent protein aggregation. The glycine substituent at position 3 would not be expected to alter the sensitization behavior, and thus compound A itself may well act as an endogenous photodamaging agent. (It must be noted, however, that while 2-amino-3-hydroxyacetophenone is a sensitizer, its *O*-β-D-glucoside, isolated by ethanolic potash extraction from human lens insoluble protein fraction, is not only photodynamically inert, but may also act as an antioxidant [37,38]).

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